THE RELATION OF *p*-AMINOBENZOIC ACID TO THE MECHANISM OF THE ACTION OF SULPHANILAMIDE.

D. D. WOODS.*

From the Department of Bacterial Chemistry (Medical Research Council), Bland-Sutton Institute of Pathology, and from the Courtauld Institute of Biochemistry, Middlesex Hospital, London, W. 1.

Received for publication February 23, 1940.

IN the preceding paper Fildes (1940) discusses a general hypothesis that disinfectants and other substances preventing the growth of bacteria act by interfering in some way with substances essential to the growth of the organism. In the present work the mode of action of sulphanilamide is investigated from the point of view of this general hypothesis.

A large number of substances known to be associated with bacterial metabolism were tested to determine whether they had an antagonistic relation to sulphanilamide akin to that of –SH and Hg but without conclusive results. While this work was in progress, Stamp (1939), working on a similar hypothesis, found that extracts of streptococci were able to antagonize the action of sulphanilamide and later, while the present paper was actually in preparation, Green (1940) obtained a preparation from *Brucella abortus*. Following Stamp's procedure it was found that yeast extracts contained a substance which, like that of Stamp and Green, reversed the inhibitory action of sulphanilamide. The chemical properties of this substance and its behaviour in growth tests indicated that it might be chemically related to sulphanilamide itself. As a result of this suggestion *p*-aminobenzoic acid was tested and found to have high anti-sulphanilamide activity. A preliminary report of this work has already been given (Woods and Fildes, 1940). The bearing of these results on the possible mode of action of the drug is discussed.

BACTERIOLOGICAL TECHNIQUE.

The organism used was *Streptococcus haemolyticus* (Richards). Stock cultures were maintained on meat infusion peptone agar and were passaged through mice every 14 days.

Medium.

The test medium was buffered Bacto-peptone (McIlwain, Fildes, Gladstone and Knight, 1939), tubed in 7 ml. lots and autoclaved, with the addition of the following materials to each tube at the time of testing :

* Halley Stewart Fellow.

	ml.
Phosphate buffer $M/18$, pH 7·6	1·175
List A (in $N/5$ HCl)	0·5
N NaOH	0·1
$M/50$ glutamine	0·125
Cocci (in peptone water)	0·1
Sulphanilamide $M/300$, or water	1·0
Test solution, or water	1·0

List A contained glucose, KH_2PO_4, aneurin, nicotinamide, β-alanine, pimelic acid and riboflavin in quantities given by McIlwain *et al.* (1939) and also 0·24 g. cystine/100 ml. The mixture was made up, stored and sterilized (by filtration) in $N/5$ HCl. The total volume of the test medium was thus 11 ml. and the final concentration of sulphanilamide $M/3300$.

At the time this work was begun the above medium was one of the simplest known on which the streptococcus will grow satisfactorily (McIlwain *et al.*, 1939). It was felt desirable to use as simple a medium as possible, as it is known that the inhibitory action of sulphanilamide is less complete in complex media containing, for example, serum or meat broth.

Inoculum.

For satisfactory testing it was necessary that sulphanilamide inhibition should be as complete as possible, and a standard of no growth for at least five days with $M/3300$ sulphanilamide was aimed at. To attain this it was found that both the size and age of the inoculum were of importance (Table I). Young cultures and large inocula both reduced the inhibitory power of the drug. As a routine therefore the inoculum chosen was *ca.* 1000 cells from a two or three day stock culture. It was also found that if the meat infusion broth used for the stock culture medium was freshly prepared inhibition was again unsatisfactory; freshly-made medium was therefore avoided. With these precautions inhibition was complete, with few exceptions, for the duration of the test (five to six days).

TABLE I.—*Effect of Size and Age of Inoculum on Sulphanilamide Inhibition.*

Cultures on meat infusion peptone agar used as source of inocula.
All tubes contained $3·03 \times 10^{-4}$ M sulphanilamide.

Age of agar cultures (hrs.).	Number of cells inoculated.	Growth after days. 1.	2.	3.	5.
24	10^6	+	+++	+++	+++
	10^5	0	+++	+++	+++
	10^4	0	0	+++	+++
	10^3	0	0	0	0
48	10^6	+	+++	+++	+++
	10^5	0	++	+++	+++
	10^4	0	0	0	0
	10^3	0	0	0	0

Here and elsewhere + signs are roughly proportional to the mass of growth. Above experiment repeated in absence of sulphanilamide gave +++ in all cases in one day.

Tests.

Activity in antagonizing the inhibitory effect of sulphanilamide was followed by determining the minimum amount of material necessary to promote full growth in the presence of a standard concentration ($M/3300$) of the drug. In each test the following series of tubes were set up: (1) medium alone (full growth normally attained in one day or less), (2) medium + sulphanilamide (in duplicate), (3) medium + top concentrations of test materials used (to test for any inhibitory or growth-stimulating effect), (4) medium + sulphanilamide + test material in falling concentration from a 1 in 5 serial dilution. Tubes were incubated in air + 5 per cent. CO_2 at 37° C. and readings taken daily for five days. It was observed throughout that if growth occurred at all, it always became maximal within 24 hours of the appearance of the first trace.

ANTI-SULPHANILAMIDE ACTIVITY OF CELL EXTRACTS, ETC.

The failure to obtain any significant activity with known essential substances led to a consideration of the possibility that the substance postulated might be one whose importance in cell metabolism had not yet been recognized and a survey was made of cell extracts, etc. At this point Stamp (1939) demonstrated that extracts of streptococcal cells contained a substance which powerfully antagonized the inhibitory action of sulphanilamide and the related drug M. & B. 693. As Stamp points out, this substance, which appears normally to be present in the cell, may be the metabolite with which the action of sulphanilamide is concerned. By applying Stamp's method of extraction (incubation in $N/25$ NH_3 at 37° C.) to baker's yeast we were able to obtain

TABLE II.—*Anti-Sulphanilamide Activity of Cell Extracts, etc.*

Activity expressed as minimum volume required to give growth in $3{\cdot}03 \times 10^{-4}$ *M* sulphanilamide.

Material.	Method of extraction.	Concentration (g./ml. extract).	Activity.
Baker's yeast	Stamp	3·5	0·0016
Yeast wash	..	≡ 3·5	0·0016
Brewer's yeast	Stamp	3·5	0·04
,, ,,	H_2O, 100° C.	3·5	0·04
,, ,,	Na_2SO_4*	3·5	1·0
Ox muscle	H_2O, 100° C.	1·0	0·2
,, ,,	Tryptic digest	1·7	0·2
,, liver	H_2O, 100° C.	1·0	0·2
Turnip	,,	1·7	0·2
Urine	..	..	0·04
Serum (horse)	..	..	0·04
Laked blood, 50 per cent. (ox)	..	..	0·2
Albumin (egg)	HCl hydrolysed	0·1	<1·0

* Method of Deutch, Eggleton and Eggleton (1938).

more active preparations than by other methods. Such extracts were also more active than the other cell extracts tried (Table II), and seemed to be a suitable starting-point for larger scale work. Less active extracts were obtained if the yeast was washed prior to extraction, and it seemed possible that a greater amount of factor might be obtained from the medium on which the yeast had been grown. A sample of yeast wash* was tested (after precipitation of inactive material with alcohol), but was found to have no higher activity (on basis of weight of yeast obtained from it) than the yeast extracts. It also contained a greater bulk of inactive material.

FRACTIONATION AND CHEMICAL PROPERTIES OF YEAST EXTRACT.

Preparation of Yeast Extract.

The procedure of Stamp for streptococci was modified to some extent for experimental convenience; in particular the incubation time was reduced from 24 to 5 hours, as this resulted in no decrease of activity and there was less trouble with contamination by anaerobes.

7 lb. fresh baker's yeast (D.C.L.) were crumbled into 5250 ml. $N/25$ NH_3 previously warmed to 43° C. and stirred till evenly suspended; the pH was then *ca.* 7·8. The suspension was incubated five to six hours at 37° C. with occasional shaking; the pH normally fell to 5·5–6. The bulk of yeast was removed by filtration through pulp, and the remainder with the Sharples centrifuge. The clear supernatant (5300 ml.) was evaporated *in vacuo* to 200 ml. and 9 volumes of alcohol added. The ppt. (inactive) was discarded. The filtrate was taken to dryness *in vacuo* and yielded *ca.* 20 g. of a thick brown syrup which was taken up in water, adjusted to pH 7, diluted to 180 ml., and filtered from a small amount of insoluble material. This solution will be referred to as Extract A. For testing it was diluted 1 in 5 to a concentration of 3·5 g. original yeast/ml. (referred to throughout as "standard strength"). Extract A is thus 5 × "standard strength." The average anti-sulphanilamide activity of several batches of Extract A was *ca.* 30 μg. dry wt./11 ml. medium (i.e. 3 parts per 10^6 medium).

Preliminary Purification of Extract.

The active material was precipitated by mercuric acetate but not by phosphotungstic acid (Table III). The latter reagent precipitated some inactive material, and a combination of the two methods removed most of the colour and reduced the dry weight of material by 50 per cent. without serious loss of activity.

Mercury precipitation.—160 ml. Extract A were treated with an excess of mercuric acetate (130 ml. 30 per cent.). The mixture was kept overnight at 0° C. and filtered. The ppt. was washed twice by resuspension in 50 ml. water containing a little mercuric acetate, decomposed with H_2S and filtered. The filtrate and two washings of the HgS ppt. were combined, aerated to remove

* I am indebted to British Fermentation Products, Ltd., for the gift of this material.

H_2S, neutralized, and evaporated to 160 ml. The original Hg filtrate was worked up in the same way for testing.

Phosphotungstic acid precipitation.—160 ml. of the decomposed Hg ppt. (as above) were acidified with 40 ml. 5*N* H_2SO_4 and an excess of 20 per cent. phosphotungstic acid in *N* H_2SO_4 added (130 ml.). After 4½ hours at 0° C. the ppt. was filtered off and washed twice by resuspension in *N* H_2SO_4 containing 1 per cent. phosphotungstic acid. Filtrate and washings were treated with $Ba(OH)_2$ until no further ppt. was obtained, and excess of $Ba(OH)_2$ was present. The filtrate (and two washings of the Ba ppt.) were slightly acidified with H_2SO_4 to remove excess Ba and refiltered. The filtrate was neutralized and evaporated *in vacuo* to 160 ml. This solution will be referred to as Extract B and is again equivalent to 5 × "standard strength."

The original phosphotungstic ppt. was decomposed by solution in warm acetone and treating with $Ba(OH)_2$. It was also tested.

The dry wt. of Extract B was *ca.* 50 mg./ml., of which 25 mg. were ash. The average activity was 16 µg./11 ml. or 1·5 parts/10^6 medium. Extract B was used for most of the following work.

Chemical Properties of the Factor.

(a) *Stability.*

The activities of Extracts A and B were unaffected by boiling, autoclaving at 15 lb. for 15 min. or heating to 100° C. for 30 min. with 0·2 *N* HCl or NaOH. It was also stable to boiling for some hours with 50 per cent. HCl. The extracts did not lose activity on storage at 0° C. for three months.

TABLE III.—*Precipitation of Factor with Metals, etc.*

Activity is expressed as the reciprocal of the minimum volume of test solution of "standard strength" (i.e. = 3·5 g. original yeast/ml.) required to give growth with 3·03 × 10^{-4} *M* sulphanilamide.

Reagent.	Activity.			
	Original solution.	Ppt.	Filtr.	Ratio: ppt./filtr.
Mercuric acetate	625	625	25	25/1
,, ,,	625	625	25	25/1
Phosphotungstic acid	625	25	625	1/25
,, ,,	125	5	125	1/25
Basic lead acetate	625	125	625	1/5
Silver nitrate, pH 2	625	5	625	1/125
,, ,, pH 7	625	625	125	5/1
,, ,, ,,	625	625	25	25/1
,, ,, ,,	125	25	25	1/1

(b) *Precipitation by metals, etc.*

Results are given in Table III. Precipitation with Hg and non-precipitation with phosphotungstic acid were clear cut and consistently obtained,

and were therefore useful in routine purification. Ag at pH 7 gave variable results ; the precipitation appeared to be less complete with weaker preparations. The separation with basic lead acetate was not sufficiently distinct for practical use.

(c) *Solvent extraction.*

The active material was extracted by ether from weakly acid (pH 4·5), but not from weakly alkaline (pH 8·5) solution (Table IV). The substance was completely recovered from ethereal solution by extraction with 1 per cent. Na_2CO_3. With butyl alcohol the factor was extracted completely at pH 3·5, and only partially at pH 7. These results indicate that the factor has weak acidic properties. The factor was extracted from weak acid solution by ethyl acetate but not by petrol ether.

TABLE IV.—*Extraction of Factor from Aqueous Solution with Various Solvents.*

Activity expressed as in Table III. The aqueous solution was extracted 5 times with an equal volume of solvent.

Solvent.	pH of aqueous phase.	Activity. Original solution.	Extract.	Residue.	Ratio: Extract/residue.
Ether	2·0	625	125	<1	>125/1
,,	4·5	625	625	5	125/1
,,	8·5	625	<1	625	<1/125
Butyl alcohol	3·5	625	625	5	125/1
,, ,,	7·0	625	125	125	1/1
Ethyl acetate	4·0	125	125	1	125/1
Petrol ether	4·0	125	<1	125	<1/125

(d) *Esterification.*

The presence of an acidic group was confirmed by the formation of an ethyl ester. The activity of the esterified material was very slight, but on hydrolysis the activity of the original extract was recovered (Table V). 20 ml. Extract B was brought to pH 4 and extracted with ether to obtain the free acid. The dried material was refluxed four hours with 1 per cent. HCl in ethyl alcohol. Alcohol and HCl were removed, the residue taken up in 20 ml. water and a 4 ml. sample of this esterified material removed for testing. The remainder was brought to pH 8·5 with $NaHCO_3$ and esters separated from any unchanged acid by ether extraction. The ether extract was evaporated, the residue hydrolysed by boiling 1½ hours with 50 per cent. (by vol.) HCl, taken to dryness, dissolved in water, neutralized and tested.

(e) *Treatment with nitrous acid.*

Activity was almost completely destroyed by mild treatment with HNO_2 in dilute aqueous solution, and this result suggests that the factor may also contain an amino group (Table V). 45 mg. barium nitrite was added to 2 ml.

Extract B at 0° C. and the mixture brought to pH 3 with H_2SO_4; after standing overnight at 0° C. it was brought to room temperature and finally to 37° C. for an hour. The solution became orange. Excess HNO_2 was removed by urea (22 mg.) and $BaSO_4$ centrifuged off.

TABLE V.—*Other Chemical Properties of the Factor.*

Activity expressed as in Table III.

Treatment.	Activity. Original material.	Product.
Nitrous acid	625	5
Acetylation	125	<1
Hydrolysis of acetylated product	..	125
Ethyl esterification	125	5
Hydrolysis of esterified product	..	125
Norite adsorption	625	1
Combined Na_2CO_3, alcohol, and alcohol-pyridine eluates of norite	..	5
Steam distillation	125	<1

(f) *Acetylation.*

Confirmatory evidence for the presence of an amino group was obtained from the observation that acetylation of the extracts led to loss of activity, and that the original activity was regenerated by hydrolysis of the acetylated product (Table V). These results might also, of course, indicate the presence of an OH group. 20 ml. Extract B were taken to dryness and dried over P_2O_5. 3 ml. pyridine and 2 ml. acetic anhydride were added, the mixture heated to 100° C. for 10 hours and then taken to dryness. Traces of pyridine were removed by dissolving in alcohol and re-evaporating. The acetylated material was taken up in water, neutralized, diluted to 20 ml. and 10 ml. taken for testing. The remainder was hydrolysed by saturating with HCl and boiling for 1½ hours.

(g) *Other properties.*

The active material was strongly adsorbed by norite charcoal (0·3 g./3 ml. Extract B). Elution with 1 per cent. Na_2CO_3, acid-alcohol and 10 per cent. pyridine in alcohol recovered only traces of the factor (Table V). The factor was not volatile in steam from acid solution. An alcohol extract of dried Extract A had equal activity to the original solution.

Survey of Chemical Properties.

The factor has weakly acidic properties [(c) and (d)]. The acid pK (*ca.* 4–5) indicated by the extraction experiments is strong evidence of the carboxylic nature of the acidic group, and is suggestive of unsubstituted fatty acids and aromatic carboxylic acids. The additional presence of an amino group [(e) and (f)] indicates that the factor is an ampholyte. The solubility in ether

excludes, in general, any ampholyte that exists in solution as a zwitterion (e.g. aliphatic or aliphatic side-chain amino acids). This in conjunction with the relatively weak acid dissociation suggested that the factor might be of the type of an amino derivative of an aromatic carboxylic acid. Purified factor preparations (acid ether extract and hydrolysed esterified material) produced a red colour on diazotizing and coupling with dimethyl-α-napthylamine ; this confirms the presence of a substance containing an aromatic amino group, but does not prove that this substance is the active substance. If the factor is indeed an amino derivative of an aromatic carboxylic acid (there are, of course, other possibilities), it would bear some chemical relationship to sulphanilamide itself. This will be discussed further in the next section in connection with evidence of a different type tending to the same conclusion.

PROPERTIES OF THE FACTOR IN GROWTH TESTS.

Concurrently with the chemical fractionation some experiments on the possible mode of action of the anti-sulphanilamide factor were carried out with partly purified material.

TABLE VI.—*Titration of Anti-Sulphanilamide Activity of Yeast Extract A.*

	ml. extract A.	Growth after hours and days. 18.	42.	3.	5.
Medium alone . . .	.	+	+++	+++	+++
,, + sulphan. (duplicate)	.	0	0	0	0
,, 0 + Extract A	1·0	+++	+++	+++	+++
,, 0 + ,,	0·2	++	+++	+++	+++
,, + ,, + ,,	1·0	+++	+++	+++	+++
,, + ,, + ,,	0·2	+++	+++	+++	+++
,, + ,, + ,,	0·04	tr.	+++	+++	+++
,, + ,, + ,,	0·008	0	tr.	+++	+++
,, + ,, + ,,	0·0016	0	0	+++	+++
,, + ,, + ,,	0·0003	0	0	0	0

Table VI shows the results of a typical titration for determination of activity. The following points may be noted :

(1) The extract has some growth-promoting activity at the higher concentrations. This type of activity became reduced with increasing fractionation, although anti-sulphanilamide activity remained unchanged.

(2) The bacteriostatic nature of sulphanilamide inhibition is confirmed. With the smaller amounts of factor the organism grew up after 2–3 days' incubation with sulphanilamide during which time no visible growth occurred.

(3) In the titration each tube contains 1/5 of the amount of factor in the preceding tube and this is the limit of accuracy of the method. If finer differences are used the results are less consistent.

It was soon evident from the sharpness of the chemical fractionation obtained with many of the precipitants and solvents used that the factor was probably a single substance and not a mixture of components each accelerating

growth. This is important, as it is known that vigorous growth, particularly during the early stages, diminishes the inhibitory effect of sulphanilamide in a non-specific manner.

It was first considered whether the factor might be working by reacting chemically with the sulphanilamide, and thus removing it from the medium. In this case it would be expected that the molar concentration of the factor would be of the same order as that of the sulphanilamide used. Comparison of the dry weight and activity of even crude factor preparations showed that this cannot be the case. 0·003 mg. of an acid ether extract of Extract B was sufficient to reverse the inhibitory action of 1 ml. 0·0033 *M* sulphanilamide. If the factor is equimolar with sulphanilamide, then 0·003 mg./ml. must be equivalent to 0·0033 *M* factor, and from this the mol. wt. of the factor works out at *ca.* 1. As even this result was obtained by assuming a crude preparation to be pure factor, it is certain that any molecular reaction can be excluded. It remained possible that the factor might act catalytically in promoting the removal of sulphanilamide.

In other experiments it was found that if the concentration of sulphanilamide is increased, it is necessary to raise the concentration of factor in the same proportion in order to reverse the inhibition. This constant quantitative relationship has been demonstrated with a number of different factor preparations at various stages of purification and also using *Bact. coli* in place of the streptococcus as the test organism. A selection of such experiments is given in Table VII. The fact that the concentration ratios are *exactly* constant is due to the fact that sulphanilamide and factor were each used in 1/5 falling concentration. As the titration cannot be used accurately with finer differences in amount of factor, it was considered better to carry out a number of separate titrations than to attempt to work with smaller differences. Sulphanilamide is insufficiently soluble for work with higher concentrations than those recorded and smaller amounts tend to give incomplete inhibition.

TABLE VII.—*Relation between Concentration of Sulphanilamide and Concentration of Factor Required to Reverse the Inhibition.*

Quantities of Extracts A and B are expressed as ml. " standard strength."

Organism.	Streptococcus.		*Bact. coli.*†		Streptococcus.	
Factor used :	Extract A.		Extract B.		*p*-aminobenzoic acid.	
Conc. sulphan. ($M \times 10^{-3}$). (*a*).	ml. required ($\times 10^{-1}$). (*b*).	Ratio :* (*b*) / (*a*).	ml. required ($\times 10^{-1}$). (*c*).	Ratio :* (*c*) / (*a*).	Conc. required ($M \times 10^{-7}$). (*d*).	Ratio: (*d*) / (*a*). ($\times 10^{-4}$).
0·303	0·016	5·3	0·08	26·5	0·58	1·92
1·515	0·08	5·3	0·4	26·5	2·91	1·92
7·575	0·4	5·3	2·0	26·5	14·54	1·92

* These arbitrary ratios are an expression of the ratio : conc. factor required/conc. sulphanilamide used.

† The strain of *Bact. coli* and the medium employed (lactate + inorganic salts) were those used by Fildes (1940).

As there was no possibility of a direct molecular reaction between sulphanilamide and the factor, this constant quantitative relationship between inhibitor

and active substance was reminiscent of the competitive inhibition of enzyme reactions by substances chemically related to the substrate or product. From this point of view the factor would be considered to be the substrate (or product) of the enzyme reaction in question and sulphanilamide the substance of related chemical structure inhibiting the reaction. It will be recalled that consideration of the chemical properties of the factor also indicated the possibility that the factor might be chemically related to sulphanilamide. Examples of known cases of competitive inhibition of enzyme reactions by substances related to the substrate or products are: (*a*) succinic dehydrogenase by malonic acid, (*b*) lipase (hydrolysis of ethyl butyrate) by acetophenone and other non-polar compounds containing a carbonyl group, and (*c*) invertase by α and β-galactose and β-*l*-arabinose. Other examples and full references to those quoted are given by Haldane (1930).

As it was possible to interpret these two distinct lines of evidence (chemical properties and behaviour in growth tests) in the same way, it seemed worth while at this stage to test for anti-sulphanilamide activity some compounds which are structurally related to sulphanilamide, and whose properties are reasonably in accord with those of the yeast factor.

ANTI-SULPHANILAMIDE ACTIVITY OF *p*-AMINOBENZOIC ACID.

In view of the probability that the acid group of the factor was carboxylic the first substance tested was *p*-aminobenzoic acid. In this acid the *p*-NH_2 of sulphanilamide is unchanged but $-SO_2NH_2$ is replaced by –COOH. *p*-aminobenzoic acid proved to have very high activity, and a final concentration of $1{\cdot}2–5{\cdot}8 \times 10^{-8}$ *M* was sufficient to reverse the inhibition caused by $3{\cdot}03 \times 10^{-4}$ *M* sulphanilamide (i.e. 0·02–0·1 μg. in 11 ml. medium compared with 570 μg. sulphanilamide). The results of a number of quantitative determinations are given in Table VIII. The agreement between separate determinations (maximum variation by a factor of 5) is satisfactory in view of the high dilutions used and possible differences in the state of the inoculum. Duplicates in the same determination did not show this variation.

TABLE VIII.—*Anti-sulphanilamide Activity of* p-*Aminobenzoic Acid.*

Sulphanilamide conc. = $3{\cdot}03 \times 10^{-4}$ *M*.

Sample of *p*-aminobenzoic acid.		Commercial.	5 × recrystallized.	From acetyl derivative.	All.
Number of determinations:					
Total . .		7	5	3	15
	Molarity				
Titrating to .	$2{\cdot}91 \times 10^{-7}$.	0	1	0	1
,, .	$5{\cdot}82 \times 10^{-8}$.	4	2	2	8
,, .	$1{\cdot}16 \times 10^{-8}$.	3	2	1	6

Purity of p-*aminobenzoic acid.*

Table VIII also shows that the activity of *p*-aminobenzoic acid is unaffected by recrystallizing five times, or by acetylating and recovering the acid by

hydrolyzing the recrystallized acetyl derivative. The two purified specimens both melted at 186° C. uncorr. (literature, 186–7° C.) and gave the following analytical figures (Weiler, Oxford):—(*a*) 5 × crystallized: C, 61·38; H, 5·15; N, 10·55 per cent.; (*b*) acetylated and hydrolyzed: C, 61·48; H, 5·08; N, 10·52 per cent. (calculated: C, 61·31; H, 5·11; N, 10·22 per cent.). From the method of preparation the most likely impurities are the *o*- and *m*-isomers and *p*-nitrobenzoic acid; these were tested (Table IX) and found to be inactive, or very feebly active compared with *p*-aminobenzoic acid. (It is equally likely that the *m*-isomer is contaminated with traces of the active *p*-compound.) There is little doubt, therefore, that the active substance is *p*-aminobenzoic acid and not an impurity in the specimen used.

Quantitative relationship with sulphanilamide.

As with the yeast factor, there was a constant relationship between the concentration of sulphanilamide used and the concentration of *p*-aminobenzoic acid required to reverse the inhibition (Table VII). In order to avoid the variation in titration found in different experiments (see above), it is necessary that the titrations be carried out in the same experiment and with the same source of inoculum and serial dilution of factor. In a number of separate experiments with the same concentration of sulphanilamide (Table VIII) the ratio molar conc. sulphanilamide/molar conc. *p*-aminobenzoic acid required ranged from 5000–25,000, reflecting the usual factor of 5 variation.

Experiments with Bact. coli.

The action of *p*-aminobenzoic acid was also demonstrated with *Bact. coli* as test organism and an entirely synthetic basal medium of lactate and inorganic salts (Fildes, 1940). On this medium the usual amount of sulphanilamide ($3{\cdot}03 \times 10^{-4}$ *M*) inhibited completely for five days, and the inhibition was overcome by the same amount of *p*-aminobenzoic acid ($5{\cdot}8 \times 10^{-8}$ *M*) as was effective with the streptococcus. This was also the case when the activity of a particular yeast extract was measured with the two organisms.

Experiments with M. & B. 693.

Under the test conditions used in this work 2-(*p*-aminobenzenesulphonamido) pyridine (M. & B. 693) has approximately five times the inhibitory activity of sulphanilamide. *p*-Aminobenzoic acid also reverses inhibition by this drug, although five times as much is required as for a similar concentration of sulphanilamide. Similar results are obtained with the yeast factor.

	p-aminobenzoic acid ($M \times 10^{-3}$).	Extract A (ml. standard strength).
Sulphanilamide ($3{\cdot}03 \times 10^{-4}$ *M*)	5·8	0·0016
M. & B. 693 ,,	29·1	0·008

Sulphanilic acid ($0{\cdot}9 \times 10^{-3}$ *M*) also inhibits, though less efficiently, and this is again reversed by *p*-aminobenzoic acid.

Growth-promoting activity.

p-aminobenzoic acid had no significant effect on the rate or mass of growth under the test conditions. There was slight acceleration at high concentrations ($0{\cdot}2 \times 10^{-3}$ *M*), but above this there was slight inhibition. No growth stimulation was found with *Bact. coli* on the ammonium lactate medium which is certainly free from preformed *p*-aminobenzoic acid. The crude yeast extracts had considerable growth-accelerating action, but this was greatly diminished as the factor was purified. The anti-sulphanilamide factor (" P ") obtained from *Brucella abortus* by Green (1940) was reported to have high growth-promoting activity for a number of organisms, but the material had not at that time been much purified.

ANTI-SULPHANILAMIDE ACTIVITY OF SUBSTANCES RELATED TO *p*-AMINOBENZOIC ACID.

Materials.

p-*acetaminobenzoic acid* was prepared from *p*-aminobenzoic according to Ritsert and Epstein (1904). The product was washed with dil. HCl and recrystallized from 20 per cent. alcohol; M.P. 250° C. The *p*-aminobenzoic acid was regenerated by refluxing two hours with conc. HCl, taking to dryness, dissolving in water and bringing to pH 4·5 with sodium acetate. After three

TABLE IX.—*Anti-Sulphanilamide Activity of Substances Related to* p-*Aminobenzoic Acid.*

Conc. of sulphanilamide = $3{\cdot}03 \times 10^{-4}$ *M*.

Substance.	Active at *M* conc.
p-aminobenzoic acid	$1{\cdot}2$–$5{\cdot}8 \times 10^{-8}$
o-aminobenzoic acid	—
m-aminobenzoic acid	$0{\cdot}9 \times 10^{-3}$
p-nitrobenzoic acid	$1{\cdot}8 \times 10^{-4}$
p-acetaminobenzoic acid	$1{\cdot}8 \times 10^{-4}$
ethyl *p*-aminobenzoate (Benzocaine)	$3{\cdot}6 \times 10^{-5}$
Novocaine	$5{\cdot}8 \times 10^{-8}$
p-hydroxybenzoic acid	—
p-toluic acid	—
benzoic acid	—
benzamide	—
p-aminobenzamide	$1{\cdot}4 \times 10^{-6}$
2-(*p*-aminobenzylamino) pyridine	$0{\cdot}9 \times 10^{-3}$
p-hydroxylaminobenzoic acid*	$5{\cdot}8 \times 10^{-8}$
p-aminophenol	—†
p-aminophenylarsonic acid (arsanilic acid)	—
sulphanilic acid	—‡

— Indicates substance inactive at 10^{-3} *M*.
* It is difficult to be sure that this compound is free from traces of *p*-aminobenzoic acid.
† Inhibits growth down to $3{\cdot}6 \times 10^{-5}$ *M*.
‡ Inhibits growth at 10^{-3} *M*.

recrystallizations the acid gave the M.P. and analytical figures already quoted (p. 84).

p-*aminobenzamide.*—*p*-nitrobenzoic acid was converted to *p*-nitrobenzamide and the latter reduced to *p*-aminobenzamide by the method of Reichenbach and Beilstein (1864). M.P., 180° C.; N, 20·4 per cent. (calc.: 20·5).

p-*hydroxylaminobenzoic acid* was prepared as an aqueous solution according to Goldschmidt and Larsen (1910), and the solid obtained and purified by the method given for the *o*-isomer by Bamberger and Pyman (1909). The substance, which is almost white when freshly prepared, darkens on exposure to light and becomes oxidized in alkaline solution. For testing, the solution (pH 7·8–8) was made up just before sterilizing and used immediately. It was kept in the dark during manipulations.

Other substances used were obtained commercially.

Results.

In general both carboxylic and amino groups in *p*-positions to one another appear to be necessary for anti-sulphanilamide activity (Table IX). Acetylation of the amino group of *p*-aminobenzoic acid leads to a ten-thousandfold decrease in activity, and ethyl esterification of the carboxylic group to a decrease of a thousandfold. These facts were useful in comparing the properties of *p*-aminobenzoic acid with those of the yeast factor. It has already been shown (Table V) that there is a large decrease of activity when the latter is acetylated or ethyl esterified and that the activity is regained on hydrolysis.

Novocaine (diethylaminoethylalcohol ester of *p*-aminobenzoic acid) appears to have the same order of activity as the free acid. The effect, however, was slightly delayed, so it is possible that novocaine is first hydrolysed by the organism.

p-*aminobenzamide*, which is even more closely related to sulphanilamide, is also active, though 100 times less so than *p*-aminobenzoic acid. Its chemical properties are also less in accord with the yeast factor.

p-*nitrobenzoic acid and p-hydroxylaminobenzoic acid.*—It has been reported (Mayer, 1937; Mayer and Oechslin, 1939) that *p*-nitrobenzenesulphonamide and *p*-hydroxylaminobenzenesulphonamide have greater *in vitro* inhibitory action than sulphanilamide itself, and it was suggested that both sulphanilamide and *p*-nitrobenzenesulphonamide are converted to *p*-hydroxylaminobenzenesulphonamide, which was considered to be the true inhibitor. If this is so it was thought possible that the corresponding –COOH analogues (*p*-hydroxylaminobenzoic acid and *p*-nitrobenzoic acid) might be even more active than *p*-aminobenzoic acid in antagonizing sulphanilamide. This did not seem to be the case. *p*-nitrobenzoic acid had only slight anti-sulphanilamide activity; this may be due to slight reduction by the organism to the active amino derivative. Mayer and Oechslin (1939) have also found that *p*-nitrobenzoic acid itself inhibits growth of the streptococcus *in vivo*, though, as in the present experiments, this was not demonstrable *in vitro*.

p-*hydroxylaminobenzoic acid* appeared to have comparable activity to *p*-aminobenzoic acid. It is possible that this compound contained traces of *p*-aminobenzoic acid, but improbable that all the activity can be ascribed to this. The hydroxylamino derivative is liable to oxidation at the pH of the

test, so it is difficult to be certain (*a*) that the concentration remains constant, or (*b*) whether the activity is due to the compound or to its oxidation products.

2-(p-*aminobenzylamino*) *pyridine** is the –CO.NH– analogue of M. & B. 693, and is related to the latter in the same way that *p*-aminobenzamide is related to sulphanilamide. It did not antagonize either sulphanilamide or M. & B. 693.

p-*aminophenylarsonic acid* (*arsanilic acid*) *and* p-*aminophenol* were tried from the point of view of replacing the –COOH of *p*-aminobenzoic acid by another acidic group. It was also thought that these substances might be inhibitory (since replacing –COOH by the acidic $-SO_3H$ or $-SO_2NH_2$ gives rise to an inhibitory substance). Arsanilic acid has been used in the chemotherapeutic treatment of sleeping sickness ; it was inactive either as inhibitor or anti-sulphanilamide agent with the streptococcus. *p*-aminophenol, which rapidly oxidizes under the test conditions, has considerable inhibitory action (Table IX), but as this is not reversed by *p*-aminobenzoic acid the inhibition is presumably of a different type.

POSSIBLE IDENTITY OF YEAST FACTOR WITH *p*-AMINOBENZOIC ACID.

All the properties of the yeast extract so far examined correspond with those of *p*-aminobenzoic acid. It was calculated from the relative activities of the two materials that the possible concentration of *p*-aminobenzoic acid in Extracts A and B was $1–2 \times 10^{-3}$ *M*.

Solutions of the acid of this range were therefore used in comparing precipitation by Hg and phosphotungstic acid, norite adsorption, etc., and the conditions used in the yeast experiments were closely followed. The yeast factor corresponded with *p*-aminobenzoic acid in the following properties :

(1) Stability to heat and acid and alkaline treatment.

(2) Presence of weakly acidic group, probably carboxylic (the acid pK of *p*-aminobenzoic acid is 4·9 ; cf. 4–5 deduced for the yeast factor).

(3) Probable presence of amino group. It will be recalled that purified yeast extracts gave a red colour on diazotizing and coupling with dimethyl-α-napthylamine. This colour was similar in tone to that obtained with *p*-aminobenzoic acid, and it was found that the intensity of the colour was of the same order as that produced by *p*-aminobenzoic acid of concentration equal to that calculated to be present in the extract.

(4) Acetylated and esterified with loss of activity which is regained on hydrolysis.

(5) Precipitated by mercuric acetate, but not by phosphotungstic acid.

(6) Free acid soluble in alcohol, ether and ethyl acetate, but not in petrol ether.

(7) Strongly adsorbed by norite charcoal.

(8) Similar biological activity in all respects.

Although this similarity in many different properties provides strong circumstantial evidence for identity, it must be emphasized that *p*-aminobenzoic acid has not yet been obtained from yeast and that final proof lies in an actual isolation. This is being attempted. The yeast factor may be a different substance, though probably similar in structure and properties.

* I am indebted to Messrs. May and Baker for a sample of this substance.

A POSSIBLE MECHANISM OF SULPHANILAMIDE INHIBITION.

The present investigation was based on a general working hypothesis that anti-bacterial substances act by interfering with some substance essential to the bacterial cell ("essential metabolite," Fildes, 1940). The experiments are also in accord with the suggestion, based on indirect evidence, that the interference is connected with inactivation of bacterial enzymes (Lockwood, 1938; McIntosh and Whitby, 1939), and provide strong evidence that the inactivation is due to competition for an enzyme between the essential metabolite and the inhibitor. A clearer hypothesis of the possible mode of action of sulphanilamide may now be built up and may prove useful as a basis for further work. Throughout the following argument *p*-aminobenzoic acid should be taken to include the probably related, if not identical, naturally occurring materials, such as yeast factor or Stamp factor.

In the first place it is suggested that *p*-aminobenzoic acid is essential for the growth of the organism. It is, however, normally synthesized in sufficient quantity by the present strain of streptococcus (and by *coli*), since it is not necessary to add it to a medium containing only known substances or preparations known to be free from anti-sulphanilamide activity (McIlwain, unpublished). It can also be extracted from the streptococcal cell (Stamp, 1939). On the basis of the experimental work it is next suggested that the enzyme reaction involved in the further utilization of *p*-aminobenzoic acid is subject to competitive inhibition by sulphanilamide, and that this inhibition is due to a structural relationship between sulphanilamide and *p*-aminobenzoic acid (which is the substrate of the enzyme reaction in question). Examples of similar competitive inhibition have already been quoted. It was found that the concentration of *p*-aminobenzoic acid required to overcome this inhibition is 1/5000–1/25,000 of the concentration of sulphanilamide used. The further course of events in a culture may now be considered as follows:

(1) p-*aminobenzoic acid is present preformed in the medium.* Growth occurs. As the anti-sulphanilamide factor appears to be widely distributed in small amount (Table II), this may account in part for the difficulty in getting complete inhibition on more complex media.

(2) p-*aminobenzoic acid is absent from the medium or present in insufficient concentration.* This would be the position under the test conditions used here. There are now two possibilities:

(2*a*) *The organism is unable to synthesize enough* p-*aminobenzoic acid.* Growth is therefore inhibited. This would normally be the case with the streptococcus under the conditions used in the present experiments.

(2*b*) *The organism is able to make sufficient* p-*aminobenzoic acid.* In this case the competitive inhibition is overcome and growth occurs. This is presumed to be the case with organisms that are insensitive to sulphanilamide.

The conditions determining whether (2*a*) or (2*b*) shall take place are delicately balanced, and this may explain why different organisms, and even the same organism under differing growth conditions, exhibit many degrees of sensitivity to sulphanilamide. It is here suggested that such differences in sensitivity are correlated with quantitative differences in ability to synthesize

p-aminobenzoic acid. On meat infusion broth (in which its growth rate approaches optimum) *Bact. coli* is almost indifferent to sulphanilamide, whilst on the ammonium lactate medium (where the growth rate is sub-optimal) inhibition is well marked. Similarly, complete inhibition of streptococcal growth is not obtained (*a*) with rich media on which growth is very rapid, or (*b*) with poorer media if the inoculum is large or consists of young actively dividing cells (Table I). To account for such variability it is suggested that the original inoculum contains sufficient *p*-aminobenzoic acid to reverse the inhibition and permit some (non-visible) growth to take place ; such growth is known to occur in the early stages of inhibition. When this supply of *p*-aminobenzoic acid becomes exhausted by the further enzyme reaction under discussion, subsequent growth will depend on the rate at which more can be synthesized, and this in turn on the number of organisms present and thus on the initial growth rate. Presence of precursors of *p*-aminobenzoic acid in the medium may also influence the rate of synthesis.

Another possible interpretation of the experimental results is that sulphanilamide inhibits the enzyme reaction involved in the *synthesis* (and not, as above, the further utilization) of *p*-aminobenzoic acid, and that it does so this time by virtue of its chemical similarity to the product of the reaction. The balance of evidence is against this view, as in this case it would be expected, on any simple interpretation, that the addition of just sufficient *p*-aminobenzoic acid for the needs of the organism should cause growth ; the amount of *p*-aminobenzoic acid needed should thus be independent of sulphanilamide concentration.

SUMMARY.

(1) Yeast extracts contain a substance which reverses the inhibitory action of sulphanilamide on the growth of haemolytic streptococci.

(2) Examination of the chemical properties of this substance and its behaviour in growth tests suggested that it might be chemically related to sulphanilamide.

(3) *p*-aminobenzoic acid has high activity in antagonizing sulphanilamide inhibition.

(4) There is strong circumstantial evidence that the yeast factor may be *p*-aminobenzoic acid.

(5) On the basis of these results a suggestion is put forward regarding the possible mode of action of sulphanilamide.

The work was carried out at the suggestion of Dr. P. Fildes, to whom I am deeply indebted for advice and criticism throughout. I am grateful to all members of the Department and to Dr. G. M. Richardson for suggestions and to Mr. D. E. Hughes for valuable assistance with some of the chemical work.

REFERENCES.

BAMBERGER, E., AND PYMAN, F. L.—(1909) *Ber. dtsch. chem. Ges.*, **42**, 2306.
DEUTCH, A., EGGLETON, M. G., AND EGGLETON, P.—(1938) *Biochem. J.*, **32**, 203.
FILDES, P.—(1940) *Brit. J. exp. Path.*, **21**, 67.
GOLDSCHMIDT, H., AND LARSEN, H.—(1910) *Z. phys. Chem.*, **71**, 437.

GREEN, H. N.—(1940) *Brit. J. exp. Path.*, **21**, 38.
HALDANE, J. B. S.—(1930) ' Enzymes,' London (Longmans, Green & Co.).
LOCKWOOD, J. S.—(1938) *J. Immunol.*, **35**, 155.
MCILWAIN, H., FILDES, P., GLADSTONE, G. P., AND KNIGHT, B. C. J. G.—(1939) *Biochem. J.*, **33**, 223.
MCINTOSH, J., AND WHITBY, L. E. H.—(1939) *Lancet*, i, 431.
MAYER, R. L. —(1937) *Biol. méd.*, **27**, suppl. p. 74.
Idem AND OECHSLIN, C.—(1939) *C.R. Soc. Biol., Paris*, **130**, 211.
REICHENBACH, E., AND BEILSTEIN, F.—(1864) *Liebigs Ann.*, **132**, 137.
RITSERT, E., AND EPSTEIN, W.—(1904) *Chem. Zbt.*, **8**, 1587.
STAMP, T. C.—(1939) *Lancet*, ii, 10.
WOODS, D. D., AND FILDES, P.—(1940) *Chem. Ind.*, **59**, 133.

THE INHIBITION OF THE ACTION OF SULPHANILAMIDE IN MICE BY *p*-AMINOBENZOIC ACID.

F. R. SELBIE.

From the Bland-Sutton Institute of Pathology, Middlesex Hospital, W. 1.

Received for publication March 18, 1940.

MCINTOSH and WHITBY (1939), taking into consideration the lag phase, the inhibition by peptone and the variability of the anti-bacterial action of the sulphanilamide drugs, concluded that these drugs acted by blocking the vital food supply of bacteria probably by the inactivation of a bacterial enzyme, of which a vital food factor is the substrate. The theory that such an enzyme reaction is involved has attracted other workers, and has been largely based on the now well-established fact that the anti-bacterial action of sulphanilamide in bacterial cultures is inhibited by a substance or substances present in peptone and in bacterial extracts (Lockwood, 1938 ; McIntosh and Whitby, 1939 ; Stamp, 1939 ; Fleming, 1940 ; Green, 1940). Woods (1940) now finds that *p*-aminobenzoic acid inhibits the action of sulphanilamide in bacterial cultures. In the present communication it will be shown that *p*-aminobenzoic acid can inhibit the therapeutic action of sulphanilamide in mice infected with streptococci.

METHODS.

The organism used was the Richards strain *Streptococcus haemolyticus*, and the general technique of the experiments followed that of Whitby (1937). The dose of streptococci chosen for inoculation in the therapeutic experiments was the number (500,000) just sufficient to kill all the untreated mice within 24 hours. The drugs were delivered directly into the oesophagus with a metal cannula fitted to a syringe. A 10 per cent. aqueous solution of the sodium salt of *p*-aminobenzoic acid (prepared by Dr. D. D. Woods) was used, so that it could be added to doses of sulphanilamide with no increase in the